Chapter 2

Expression Vectors Used in Project-Oriented Teaching Laboratories

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Introduction

This workshop presented at the 25th ABLE conference is an update of the original workshop presented at the 17th ABLE workshop (Karcher *et al.* 1996). This ABLE workshop is part of an intensive 5 week long--two credits--laboratory module in molecular and cell biology taught in the Department of Biological Sciences at Purdue University. The laboratory, taken by juniors, seniors, and graduate students, meets once a week for a one hour lecture and twice a week for four hour long laboratory sessions. In the laboratory, students learn techniques and methods and perform a laboratory project focusing on the techniques they have learned in the module. After completing this laboratory module, students may take other 5 week long laboratory modules offered in areas such as DNA fingerprinting, DNA sequence analysis, genomic cloning, transposon mutagenesis, etc.

A brief overview and outlines of the laboratory as originally taught and in its current rendition are given below. As we first taught this multi-week, project-oriented laboratory, students performed a wide range of techniques including subcloning to transfer the cDNA of interest from one vector into the pGEX expression vector. More recently, we have modified the laboratory class. Students now perform a Bradford assay to determine the amount of protein in a sample. During the first three weeks of the class, students learn the techniques of SDS-PAGE, affinity purification, and Western blotting with a particular fusion protein. For the last two weeks of class, students work independently using the techniques they have just learned to identify an unknown fusion protein. We have a collection of 4 different *E. coli* strains containing different fusion proteins. Students are told what the four possible fusion proteins are. The students then determine which protein they have been given. For additional information about the laboratory modules, contact the authors.

Background to the Laboratory Module

The goal of this introductory module is to teach certain fundamental techniques used in modern molecular and cellular biology research. Many of the materials and reagents used in this module were developed in the research laboratory of C. J. Staiger in the Department of Biological Sciences at Purdue University. The project the students work on during this 5-week class served as a foundation for subsequent studies on the functional properties of a particular cytoskeletal protein known as actin depolymerizing factor (ADF). In this project-oriented laboratory the students learn several basic techniques including:

- DNA isolation, restriction digestion, cloning, and bacterial manipulation
- over-expression and affinity purification of proteins from *E. coli*
- analysis of proteins by SDS-PAGE and by Western immunoblotting.

The entire module utilizes a cDNA for ADF1 isolated from the model genetic organism, *Arabidopsis thaliana*. Students subclone a fragment containing the ADF1 coding region from a plasmid into the expression vector pGEX-KG (Guan and Dixon, 1991), creating an in-frame fusion between glutathione S-transferase (GST) of the expression vector and ADF1. Over-expressed fusion protein is purified by affinity chromatography on glutathione-agarose beads. The fusion is released by eluting with an excess of reduced glutathione. Alternatively, a site-specific protease is used to cleave between the two portions releasing free ADF1.

General Timetable for the Original Laboratory Module

WEEK 1 Review of sterile technique. Review of use of micropipetors. Subcloning of gene of interest into an expression vector: Restriction digestion of DNA, electrophoresis of DNA fragments. "Clean-up" of DNAs. Quantitation of DNAs by gel electrophoresis. Ligation reactions. Transformation of *E. coli* cells.

WEEK 2 Mini-prep of recombinant clones to screen for inserts; gels to analyze clones. Begin work with overexpression of protein from a clone. Determine ideal time and temperature for protein expression.

WEEK 3 SDS-polyacrylamide gel electrophoresis; analysis of results from week 2.

WEEK 4 Overexpression of protein and affinity purification. SDS-PAGE gels of proteins, and Western immunoblot.

WEEK 5 Protein gel electrophoresis and Western blot. Final project: Purification of the fusion protein by affinity chromatography and/or digestion with a site-specific protease.

General Timetable for the Current Laboratory Module

WEEK 1 Review of sterile technique. Review of use of micropipetors. Bradford assay to quantitate proteins. Introduction to casting and running vertical minigels. IPTG induction of bacterial strains. Collection of samples of induced bacteria and analysis of bacterial proteins by SDS-PAGE and Coomasie stain.

WEEK 2 SDS-PAGE and Western transfer of crude bacterial lysates from week 1. Western blot analysis.

WEEK 3 Induction of bacterial strains, lysis of bacteria, and GST-affinity purification of the overexpressed fusion protein. Elution of the affinity purified protein or thrombin digestion of the fusion protein. Gel for Western blot of affinity purified proteins.

WEEK 4 Completion of Western blot analysis of affinity purified proteins. Start of independent project-- Analysis of an unknown fusion protein using the methods learned in weeks 1-3.

WEEK 5 Continuation of analysis of unknown fusion protein.

Introduction to the ABLE Workshop

The ABLE workshop focused on one part of the 5-week class outlined above. This chapter presents the protocols to determine the optimal conditions for overexpression of a cloned fusion protein in *E. coli*, to isolate the proteins from *E. coli*, and to separate the proteins by SDS-polyacrylamide gel electrophoresis. Additional procedures to purify the fusion protein by affinity chromatography and to separate the GST protein from the recombinant protein by site-specific protease cleavage are given.

The materials presented here are used in an upper-level laboratory course at Purdue. However, the protocols could be used in an introductory level laboratory course. For such an introductory course, the student can readily perform protocols 1 and 2.

Expression vectors

In order to obtain large quantities of rare proteins, an expression vector system is often used. The cDNA clone of the gene for the protein of interest is cloned into an expression vector. Frequently, a cloned gene of interest is expressed in *E. coli,* although cloned eukaryotic genes are also expressed in mammalian tissue culture cells or using baculoviruses grown in insect cells.

For an extensive review of the use of expression vectors, see Ausubel *et al*. (1995) and Sambrook *et al.* (1989). The overexpression of the cloned gene produces adequate amounts of protein for a variety of experimental uses including the production of antibodies specific to the protein which can be used to study the location of the protein within a cell, and the production of enough protein to use in biochemical, cell biology or structural studies.

General features of an *E. coli* expression vector include an origin of DNA replication on the plasmid that functions in *E. coli*; a selectable marker, such as resistance to an antibiotic, to select for the presence of the plasmid vector; a strong, regulatable transcriptional promoter which is off under some condition and can, upon induction of the promoter, produce a large amount of mRNA from the cloned gene; translational control sequences that function in *E. coli*, the appropriately positioned ribosome binding site and initiator ATG; a series of restriction endonuclease cleavage sites (a multiple cloning site or polylinker) to simplify insertion of the gene of interest in the correct orientation or open reading frame.

One type of expression vector, a fusion vector, creates a fusion protein between a protein that can readily be identified or isolated, called a tag, and the gene product of interest. The fusion protein is isolated from other proteins in the *E. coli* cell by affinity purification of the tag protein. Examples of protein tags include the maltose-binding protein of *E. coli*, which is purified by binding to amylose; and glutathione-S-transferase, which binds to glutathione. In a fusion vector, the gene of interest is cloned into the vector to maintain the correct reading frame of the gene of interest (again the multiple cloning site is useful for this) and to create a protein fusion between the tag protein and the gene product of interest. Also in the fusion vector is a region coding for a specific protease cleavage site to allow separation of the protein of interest from the tag protein.

The expression vector, pGEX-KG (See Figure 1)

A fusion protein between GST and ADF1 is produced in *E. coli* (XL1-B) cells that contain the appropriate recombinant DNA plasmid. Expression from the pGEX-KG plasmid is under the control of a strong, inducible *tac* promoter; a hybrid of the *lac* and *trp* promoters (reviewed in Sambrook *et al*., 1989). Transcription of the fusion construct is induced with the lactose analog, isopropyl ß-D-thiogalactoside (IPTG). Transcription of the *lac* operon is normally silenced by binding of the *lac* repressor protein (the product of the *lacI* gene) to the operator region of the *lac* promoter. IPTG, like many other ß-galactosides, binds to the *lac* repressor and inhibits binding to DNA, thereby allowing transcription to occur. The pGEX series of plasmid expression vectors all contain a $lacI^q$ gene for high-level constitutive expression of the *lac* repressor, and can be grown in any *E. coli* strain.

All of the pGEX vectors permit the generation of an in-frame fusion between a cDNA of interest and the glutathione S-transferase (GST) (commercially available from Pharmacia) gene isolated from *Schistosoma japonicum*, a trematode worm that infects mammalian hosts (Smith *et al.*, 1986). Because several different restriction endonuclease sites are included in a multiple cloning site (MCS) downstream of the GST gene and because the series of pGEX vectors are designed to give all three translational reading frames, almost any cDNA of interest can be cloned into these vectors to give an in-frame fusion construct. The glutathione S-transferase portion of the recombinant fusion protein serves as a convenient "tag" for the detection and purification of the protein product. For detection purposes, both immunological and enzymatic strategies are possible. Polyclonal antisera are available commercially (see Pharmacia GST Gene Fusion Manual), and are used to detect the fusion protein construct on Western immunoblots. A second method is to use an assay in which the GST enzyme conjugates reduces glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) producing a product that can be quantified by UV spectrophotometry at 340 nm.

Glutathione S-transferases are a family of detoxifying enzymes that protect cells from the detrimental effects of a wide range of xenobiotic agents by conjugating reduced glutathione to these molecules (reviewed in Mannervik, 1985). Many GST isozymes also protect cells from oxidative damage caused by hydrogen peroxide and organic peroxidases via the following reaction:

2 reduced glutathione (GSH) + R--OH ----------> GS-SG (oxidized glutathione) + H_2O + ROH

Reduced glutathione (GSH) is a tripeptide, γ-glu--cys--gly, with a sulfhydryl-containing cysteine residue linked to the γcarbon on the side chain of glutamic acid. In some cells, such as mammalian red blood cells, glutathione is present at cellular concentrations up to 5 mM and functions as a sulfhydryl buffer (Stryer, 1995). This allows many cellular proteins, such as hemoglobin, to maintain cysteine residues in the reduced state, which is often necessary to maintain protein function.

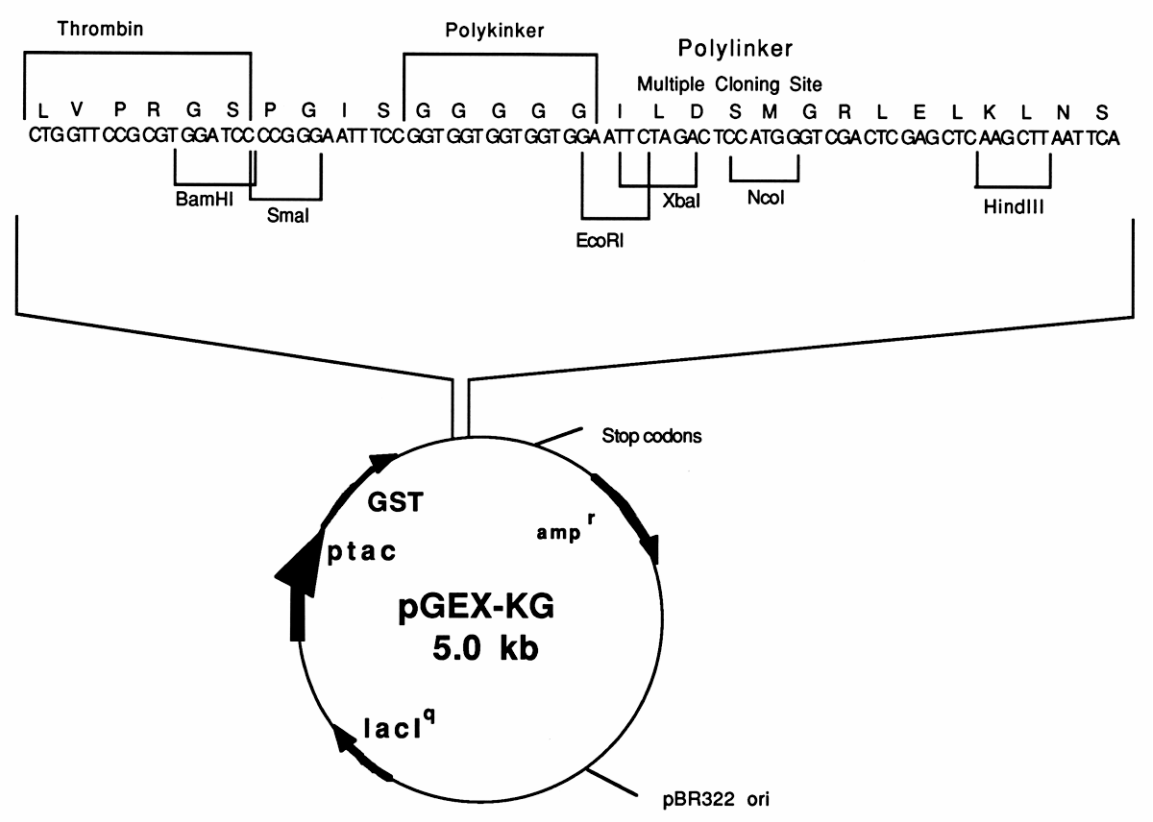

Figure 1. The expression vector pGEX-KG (Guan and Dixon, 1991). The vector uses a pBR322 origin of DNA replication. The ampicillin resistance gene allows for the selection of the presence of this vector. GST is the gene for glutathione S-transferase, which serves as a tag for purification of the fusion protein. The transcriptional promoter used to direct expression of the GST protein or of a fusion protein is p*tac*, which contains elements of the *E. coli* promoters for *lac* and *trp*. Transcription from the *tac* promoter is regulated by the lac repressor (the *lacI* gene product). The presence of a lactose analog, such as IPTG prevents binding of the lac repressor to the p*tac* promoter and allows transcription of the gene to occur. *lacI*^{q} is the gene for the lac repressor that is expressed at very high levels. (q refers to quantity.) The presence of the *lacI*^{q} gene on the pGEX-KG plasmid assures that the *tac* promoter will not be transcribed until induced by IPTG. The multiple-cloning site contains different restriction endonuclease sites that are used to clone the gene of interest in the correct open reading frame to create a fusion protein. The thrombin site is the sequence where the site-specific protease, thrombin, can cleave to separate the GST tag protein from the recombinant polypeptide of interest. The polykinker site is the sequence that codes for a series of 5 glycine residues. These glycines provide greater flexibility in the polypeptide chain and allow thrombin cleavage to occur while the GST moiety is bound to reduced glutathione on an affinity column.

The ability of GST to bind to and to use reduced glutathione as a substrate for the reduction of many different compounds is exploited for its purification. Reduced glutathione can be covalently coupled to agarose or Sepharose beads. These glutathione-beads then provide a matrix for "substrate affinity purification." This is done in one of two ways; by immobilizing the beads on a chromatography column and passing an extract containing GST over the column matrix, or by adding

a suspension of the beads to an extract or a bacterial lysate and after a short incubation period, collecting the beads by centrifugation. In both situations, the GST and GST fusions will bind with high affinity to the glutathione agarose and will be depleted from the crude bacterial extract. (See Figure 2.)

In this experiment (Protocol 1), saturated, overnight cultures are used to inoculate fresh medium and the bacterial cells are grown to log-phase. IPTG is added to the cultures to induce overexpression of fusion protein (or unfused GST) and the cells are grown for an additional 1-3 hours. Bacterial cells are harvested by centrifugation, resuspended in buffer, and lysed. Total protein extracts are examined by SDS-PAGE gel electrophoresis (Protocol 2). In the additional procedures given (Protocols 3 and 4), after the bacterial lysate is cleared of unbroken cells and cellular debris, the fusion protein is purified from the supernatant using glutathione-agarose beads. Protein extracts and purified products are analyzed by SDS gel electrophoresis. They can also be analyzed by Western immunoblotting. (GST antibodies are commercially available from Pharmacia.) In addition, the purified protein is eluted from the affinity matrix using an excess of soluble reduced glutathione, and/or the ADF1 polypeptide is cleaved from GST with the site-specific protease, thrombin.

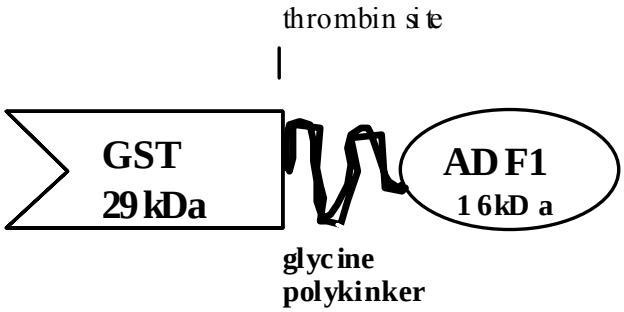

Figure 2. Schematic diagram of the recombinant fusion protein. An illustration of how the GST tag is utilized to purify the fusion protein by affinity chromatography. Reduced glutathione is covalently bound to agarose. The active site of the GST (glutathione-S-transferase) part of the fusion protein binds to the glutathione. This allows purification of the fusion protein from other proteins of *E. coli*. The fusion protein may be eluted from the affinity column by adding excess glutathione or the fusion protein may be cleaved by thrombin to release the purified protein of interest. The GGGGG polykinker allows thrombin cleavage to occur while the GST is still bound to the affinity column.

Protocol 1: Overexpression of fusion protein in *E. coli;* Preparation of total cell extracts; Determination of optimal time and temperature conditions for overexpression of the fusion protein

Introduction

Using a recombinant clone containing the gene of interest (in this case, ADF) cloned into the expression vector, pGEX-KG, over-expression of the fusion protein in *E. coli* is induced. The optimum time and temperature conditions for protein expression are determined. The results are then analyzed by SDS-PAGE gel electrophoresis.

Materials

- 100 mM IPTG isopropyl ß-D-thiogalactoside, Sigma (1-800-325-3010) catalogue number I-5502, 1 gram. Dissolve 500 mg IPTG in 20 ml of distilled water, filter-sterilize, and store frozen in 500 µl aliquots.
- 1X PBS (phosphate-buffered saline): 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.4). Make a 10X stock solution. Autoclave to sterilize the solution.
- Shaking water baths at 25°C, 30°C, 37°C
- Microcentrifuge
- 2X Laemmli protein sample buffer

  per 10 milliliters, combine:
  2.5 ml 0.5 M Tris, pH 6.8
  4 ml 10% SDS
  2 ml glycerol
  1 ml ß-mercaptoethanol
  0.01% (w/v) bromophenol blue

- *E. coli* strains, on L-agar plates with or without the antibiotic ampicillin:
  1) contains the gene of interest cloned into the expression vector pGEX-KG (ampicillin resistant); pGEX-KG with ADF1 cloned insert: Dr. Staiger; pGEX-KG:Guan and Dixon (1991); other pGEX vectors are commercially available: pGEX-2T, catalogue number 27-4805-01, Pharmacia, 1-800-526-3593
  2) contains just the expression vector pGEX-KG (ampicillin resistant)
  3) is the *E. coli* strain XL1-B (Stratagene, 1-800-424-5444, catalogue number 200268) without any plasmids (ampicillin sensitive); other *E. coli* strains can be used.

- L broth: 10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter. Sterilized by autoclaving for 20 minutes.
  For L-agar plates: add 15 g Bacto-agar before autoclaving.
  For L-agar plates plus ampicillin: After autoclaving, cool a flask of L-agar to about 45°C, then add ampicillin dissolved in sterile water to a final concentration of 100 µg/ml, before pouring plates.
- L-broth plus ampicillin at 100 µg/ml
- Ampicillin, sodium salt, Sigma (1-800-325-3010) catalogue number A-9518.
- 1.5 ml microcentrifuge tubes, sterilized by autoclaving
- Sterile test tubes in which to grow bacterial cultures
- Bromophenol blue, Sigma (1-800-325-3010) catalogue number B-1763
- Glycerol, Sigma (1-800-325-3010) catalogue number G-6279 500 ml
- ß-mercaptoethanol, Sigma (1-800-325-3010) catalogue number M-6250

Procedures

1. The day before the laboratory, grow overnight cultures of the three *E. coli* strains. Inoculate 2 ml of LB medium plus ampicillin from a plate of *E. coli* 1 (containing the cloned gene of interest) Also inoculate another tube of LB medium plus ampicillin with *E. coli* 2 (containing the pGEX-KG plasmid without insert). Additionally, inoculate 2 ml of LB without antibiotic with *E. coli* 3 (XL1-B cells that do not contain any plasmid). Grow the cultures overnight at 37°C in a shaking water bath. *E. coli* 1 contains the fusion protein to be studied; *E. coli* 2 and 3 will serve as controls.
2. Two hours before the scheduled laboratory: Using 400 µl of each of the saturated overnight cultures, set up the night before, inoculate 4 ml of fresh medium (LB+AMP for those bacterial cells containing a plasmid and LB without AMP for XL1-B cells). Make 4 tubes of the fusion protein construct, 4 tubes for the control pGEX-KG plasmid and 4 tubes of the control XL1-B cells.
3. Grow the cultures in a shaking water bath for 2 hours at 37°C. The optical density OD_{600} will be between 0.6-0.8.
4. Induce the expression of the fusion protein by adding IPTG to 1 mM final concentration. (For example, add 20 µl of a 100 mM stock.)
5. The effect of time and temperature on the overexpression of the fusion protein is determined. Collect samples of the fusion protein construct (*E. coli* 1) and the control cultures (*E. coli* 2 and 3).
    A. Time course: Following IPTG induction, grow the cells at 30°C and collect samples from all 3 strains hourly, starting at the time of induction and continuing until 3 hours after induction.
    B. Temperature: Place tubes of all 3 *E. coli* strains in shaking water baths at 25°C, 30°C and 37°C. Collect samples of cells from each tube 3 hours after induction.
6. To collect samples: Remove 0.5 ml of cells from a sample. Centrifuge the sample at maximum speed in a microcentrifuge for 30 seconds. Remove and discard the supernatant solution.
7. Resuspend the cell pellet in 200 µl of 2X Laemmli protein sample buffer. Vortex the sample to aid in the resuspension. Heat the sample for 5 minutes at 90-100°C. Allow the sample to cool. Sediment any cellular debris by centrifugation at maximum speed in a microcentrifuge for 5 minutes. Transfer the supernatant to a fresh, labeled microcentrifuge tube. Store the samples at in the freezer (-20°C) until ready to run an SDS-PAGE gel of the samples.
8. Make an SDS-PAGE gel (10% resolving gel, 3% stacking gel) according to protocol 2.
9. Load 20 µl of each sample on the SDS-polyacrylamide gel, run the gel for approximately 45 minutes to separate the proteins, and stain the gel with Coomassie Brilliant Blue (according to Protocol 2.)
10. After de-staining, examine the gel for the overexpressed fusion protein and the parental GST protein (~26 kDa). Remember that the size of the fusion protein is predicted by adding the M_r of the GST and ADF1 (~16 kDa) polypeptides. Determine which lanes contain the greatest amount of fusion protein and determine the optimal time and temperature conditions for the induction. See Figure 3 for an example of typical results.

Protocol 2 SDS-PAGE Analysis of Proteins

Introduction

The analysis of proteins is a key element in modern cell biology. The method that is most widely used is polyacrylamide gel electrophoresis (PAGE) of proteins in the presence of the ionic detergent, SDS (sodium dodecyl sulfate). For a general introduction on electrophoresis see Andrews (1986). Mordacq and Ellington (1994) discuss the set-up and running of PAGE gels.

Electrophoresis is the migration of a charged particle in the presence of an electrical field. The net charge on a protein varies with pH and is usually small. To separate proteins on the basis of differences in molecular weight, not charge, the anionic detergent, sodium dodecyl sulfate, SDS is used. SDS binds very tightly to hydrophobic regions of proteins and causes protein unfolding, and disruption of protein-protein interactions. When proteins are heated in the presence of SDS and a reducing agent with a soluble thio group to reduce disulfide bonds, such as β-mercaptoethanol or dithiothreitol, all higher order protein structure is disrupted.

In the presence of an excess of SDS, the protein backbone is coated with the negatively charged SDS and the net charge on the protein per unit mass is approximately constant. In this situation, the migration of a protein in an electric field is proportional to its size. This allows a mixture of proteins to be separated on the basis of their molecular weights in a gel matrix in the presence of excess SDS.

The molecular weight of a protein can be determined by measuring its migration through a gel relative to the migration of protein standards of known molecular weights in the same gel. The relationship $\log M_r = R_m$ applies, where M_r is the molecular weight of the protein and R_m is the relative migration of the protein.

Polyacrylamide

Typically, a polyacrylamide gel is cast in the appropriate buffer. This gel consists of a matrix of acrylamide monomer cross-linked with N,N'-methylenebisacrylamide (Bis) to form a co-polymer. Acrylamide (CH_2=CH-CO-NH_2) forms long polyacrylamide chains that are occasionally cross-linked by the bifunctional reagent Bis (CH_2=CH-CONH-CH_2-NH-CO-CH=CH_2).

The higher the concentration of Bis, the more frequent are cross-links between polyacrylamide polymers. The gaps in the matrix are called the pores. The pore size reflects the relative concentration of acrylamide and Bis. It is the pore size that determines the effective viscosity of the gel. Therefore, the higher the Bis concentration, the tighter the pore size and the slower the proteins will migrate.

To start the polymerization reaction of the acrylamide monomer and the bis crosslinker, the catalysts TEMED (N, N, N', N'-tetramethylethylenediamine) and AMPS (ammonium persulfate) are used. These two components set up a free-radical generating system. AMPS initiates the process by generating a free radical, which in turn, activates the TEMED. The activated TEMED then donates an electron to an acrylamide monomer to create an acrylamide free radical. The acrylamide free radical reacts with other acrylamide monomers or with bis to create a polymer chain.

Discontinuous SDS PAGE

Often it is useful to cast two gels--a separating gel (lower, or resolving gel) and a short stacking gel (upper gel). The separating gel and the stacking gel are of opposite acidity (usually the separating gel is alkaline, pH 8.8, and the stacking gel is slightly acidic, pH 6.8). The sample is dissolved in the stacking gel buffer and the running buffer is the same as the separating gel. This pH discontinuity causes proteins to alter their mobilities depending upon which gel they find themselves in. Effectively, this causes all of the proteins to "stack" so that they all enter the separating gel at the

same time. This is especially useful because the investigator usually has to load a significant volume of sample onto the gel. The stacking effect offsets the large volume. A good rule of thumb is that the height (volume) of the stacking gel should be twice the height (volume) of the sample. In practice, with the BioRad minigel apparatus used in this exercise, one leaves approximately 1 cm between the bottom of the wells and the resolving gel.

Dreyfuss mini-gels

These exercises use Dreyfuss gels (Dreyfuss *et al.*, 1984.) These are SDS PAGE gels cast in a vertical gel apparatus. The acrylamide to Bis ratio is held constant. However, the investigator can select from a wide range of viscosities by varying the acrylamide concentration. If very small proteins are to be separated, use a high percentage gel; if very large proteins are to be separated, use a lower percentage gel.

Materials

- 4X Lower Gel Buffer (LGB)
  1.5M TRIS-HCl pH 8.8 181.65 g/liter
  0.4% w/v SDS 4.0 g/liter

- 4X Upper Gel Buffer (UGB)
  0.5M TRIS-HCl pH 6.8 60.55 g/liter
  0.4% w/v SDS 4.0 g/liter

- Running Buffer [for 1 liter of 5X stock]; after dilution to 1X, the pH is ~8.4
  72g Glycine
  15g Tris-base
  5g SDS

- Acrylamide Solution
  30% w/v stock from BioRad (161-0158), containing a 37.5:1 ratio of acrylamide:bis-acrylamide

Caution: Acrylamide is a neurotoxin. Do not mouth pipet. Wear gloves when handling the materials. Wash hands after each use of acrylamide. The liquid form of acrylamide (monomer) is much more hazardous than the polymer.

- Molecular weight standards: SDS-PAGE standards, broad range, BioRad (1-800-424-6723) catalog number 161-0317. Use an aliquot per gel lane as directed by suppliers' instructions.

- Laemmli Sample buffer:

  2X, per 10 ml: 2.5 ml 0.5 M Tris, pH 6.8
  4 ml 10% SDS
  2 ml glycerol
  1 ml ß-mercaptoethanol
  0.01% (w/v) bromophenol blue
  5X, per 10 ml: 4 ml 0.5 M Tris, pH 6.8
  0.77 g dithiothreitol
  1 g SDS
  dissolve, then add 5 ml glycerol
  0.025% (w/v) bromophenol blue

- 10% (w/v) ammonium persulfate (AMPS); in distilled water. BioRad (1-800-424-6723) catalogue number 161-0700. The powder may be stored, desiccated, at room temperature for a year. In solution, ammonium persulfate can be stored at 4°C for approximately a week.

- TEMED (N,N,N', N'-tetramethylethyldiamine) BioRad (1-800-424-6723) catalogue number 161-0800.

- BioRad MiniProtean II mini gel apparatus; (Other vertical gel apparati may be used.) This includes: lower buffer chamber (clear) and lid (green) with attached electrode cables, casting stand with rubber gaskets, inner cooling core (white), sandwich clamp assemblies (x2), glass plates; 1 long and 1 short per gel, pair of gray spacers, Teflon comb-15 well. BioRad (1-800-424-6723) catalogue number 161-2940. MiniProtean II Cell

- Power supply: (Other suitable power supplies may be used.) Power Pac 300 Power Supply, BioRad
- (1-800-424-6723) catalog number 165-5050.

- Coomassie Brilliant Blue R 0.25% (w/v) in 40% (v/v) methanol, 7% (v/v) acetic acid. (Sigma (1-800-325-3010) catalogue number B-8647) 1 bottle is diluted with deionized or distilled water to make 1 liter of working solution to stain proteins in gels.

- Gel loading tips (These are longer and narrower at the tip than the standard tips.) Midwest Scientific 1-800-227-9997; catalogue number MPT1000.

- Micropipetor, such as the Gilson P-200. Rainin, 1-800-472-4646.

- SDS Sodium dodecyl sulfate, also called lauryl sulfate. Biorad, 1-800-424-6723, catalogue number 161-0301.

- TrisHCl, Tris{hydroxymethyl}amino-methane or Trizma, Sigma (1-800-325-3010) catalogue number T-6791 Trizma base, 100 gram $26.40 US; T-6666, Trizma hydrochloride.

- β-mercaptoethanol, Sigma (1-800-325-3010) catalogue number M-6250, 2-mercaptoethanol, 100 ml.

- Dithiothreitol, Sigma (1-800-325-3010) catalogue number D-5545.

- Glycerol, Sigma (1-800-325-3010) catalogue number G-6279.

- Glycine, Sigma (1-800-325-3010) catalogue number G-7403.

Procedure:

For additional guidelines on casting gels, see the BioRad Mini-Protean II Instruction Manual.

1. For each gel, clean a set of glass plates (one long and one short), a pair of spacers and a comb using ethanol.
2. Assemble a gel "sandwich" on a clean Kimwipe starting with the large plate. Lay each gray spacer along the short edge of the large glass plate so that the bottom edge of the plate and spacers is roughly aligned. Next, place a short glass plate on top of the spacers and align it with the bottom of the spacers and the large glass plate. The spacers will protrude above the large glass plate by about 5 mm.

3. Loosen, but do not remove, the screws on the clamp assembly and stand it on the bench with the screws facing away. Pick the gel sandwich up carefully, and with the large plate facing away, slide it into the clamp assembly. The large plate should face the acrylic pressure plate and the short plate will face outward. Tighten the top two screws of the clamp assembly.
4. Turn the clamp assembly over and make sure that the two glass plates and spacers are each aligned on the bottom edge. All three surfaces must be flush, or the gel will leak during casting. If the surfaces are not aligned, turn the apparatus over, loosen the top screws and rearrange the plates and spacers.
5. Place the clamp assembly onto the rubber gasket in the casting stand with the acrylic pressure plate closest to the center and the glass plates facing outward. Snap the acrylic plate under the overhanging arm by pushing on the white portions of the clamp.
6. Prepare an acrylamide solution for the resolving gel according to the following recipes: (Cut the amounts in half if only 1 gel is needed.)

RESOLVING GEL (10 ml, enough for 2 mini gels)

component	7.5%	10.0%	12.5%	15%
water	5 ml	4.1 ml	3.4 ml	2.5 ml
L.G.B.	2.5 ml	2.5 ml	2.5 ml	2.5 ml
acrylamide	2.5 ml	3.4 ml	4.1 ml	5 ml

7. Add 34 µl of fresh 10% AMPS and 5 µl of TEMED. AMPS and TEMED are catalysts that promote the polymerization process. Swirl the solution gently and pour immediately using a Pasteur pipet and rubber bulb. Pouring the gel is easiest if the pipet is placed against the middle of the long glass plate and the solution allowed to flow down between the two plates. Try not to introduce any air bubbles into the gel.
8. Fill to a level that is approximately 0.5 cm below the notch found on the clamp assembly, or alternatively place a comb into the gel assembly and make a mark approximately 1 cm below the teeth and fill to this level.
9. Carefully overlay the resolving gel monomer solution with water. Use a slow steady rate to prevent mixing of the water and monomer solution.
10. Allow the gel to polymerize for 30-45 min. The remaining acrylamide solution can be used as a good indicator for polymerization.

Note: It is sometimes necessary to pour a resolving gel and to store it for use on the following day. If this method is chosen, dilute some lower gel buffer (LGB) 1:4 with distilled water and use it to overlay the resolving gel after it has been thoroughly rinsed with distilled water. Wrap the gel assembly and clamp in plastic wrap and store it in the refrigerator.

11. Prepare a solution for the stacking gel according to the following recipe. For these gels, use a 3% stacking gel regardless of the % resolving gel.

STACKING GEL (5 ml, enough for 2 minigels)

component	3%
water	3.25 ml

U.G.B 1.75 ml
acrylamide 0.5 ml

12. Dry the area above the resolving gel, and dry the glass plates, with a piece of filter paper.
13. Add 23 µl of 10% AMPS and 7.5 µl of TEMED. Mix briefly. Transfer into the gel plates using a Pasteur pipet. Bring the level of solution to the top of the lower glass plate.
14. Insert a 15 well comb between the two glass plates by inserting one edge first with the comb at a 45º angle. Once all of the teeth are between the glass plates push the comb down until the arms make contact with the spacers.
15. Allow the stacking gel to polymerize for 30-45 minutes.
16. Remove the comb by pulling it straight up. The clamp assembly is then attached to the inner cooling core. The upper edge of the white portion on the clamp assembly has two appendages. These insert into slots on the upper portion of the cooling core. Make sure that the lower glass plate is positioned properly against the U-shaped gray gasket. Note that the gasket has notch approx. 1 cm from the top. The inner glass plate must make a good seal by resting against this notch, otherwise the upper buffer chamber will leak. Snap the bottom portion of the clamp assembly firmly into place by pushing at the bottom edges.
17. The upper buffer chamber is formed by placing a second gel assembly onto the opposite side of the inner cooling core. If only one gel is to be run, clamp a gel assembly without spacers (i.e. long glass plate immediately adjacent to a short plate) into the other side of the apparatus.
18. Prepare 300 ml of 1X running buffer by diluting 60 ml of 5X running buffer with 240 ml of distilled water.
19. Place the upper buffer chamber/gel assembly into the lower buffer chamber (the clear plastic tank). Add approximately 115 ml of 1X running buffer to the upper chamber. Use a Pasteur pipet to wash out the wells.
20. Fill the bottom buffer chamber with 1X running buffer until the level covers at least 1 cm of the gel.
21. Carefully load your gel samples using the narrow and long gel loading tips and a micropipetor. Use a slow, smooth action on the micropipetor and the glycerol in the loading buffer will cause the sample to drop into the bottom of the well. Also load a lane with molecular weight standards. Typically 5 or 10 microliters is used. The amount to load will be designated by the supplier.
22. Put the green lid onto the apparatus with the black electrode matching the black cable. Plug the cables into a power supply. (Check that the polarity of the leads is correct!!) Remember that proteins coated with the anionic detergent SDS will have a net negative charge and will migrate toward the positive electrode.
23. Run the gel at 150 volts for approximately 45 minutes. (This should be a current of less than 50 mA.)
24. When the tracking dye has migrated to within a cm from the end of the gel, shut off the power supply and remove the lid. Take the entire apparatus to the sink. Lift out the upper buffer chamber and gels and tip the upper buffer into the sink. Remove the gel assembly from the inner electrode core by pushing upward from the bottom edges of the gel assembly.

25. Loosen the 4 screws on the gel assembly and slide the two glass plates and gel out of the assembly.
26. Remove the two spacers by sliding them laterally.
27. Use a thin metal spatula, or a razor blade, to pry the two glass plates apart. **Do this from the middle of the plates, not at the corner, otherwise the plate may break.** The gel should remain attached to one of the glass plates.

Coomassie Blue staining: Use plastic boxes with tight-fitting covers when staining and destaining to minimize the exposure to methanol and acetic acid vapors. If available, place these boxes in a fume hood also.

28. Wear gloves when handling a gel. Grasp the gel by two corners and lift it from the plate. Place it in a small plastic staining container.
29. Cover the gel with 50 ml of 0.25 % Coomassie Brilliant Blue dissolved in 40% methanol, 7% acetic acid (the methanol fixes the proteins in the gel so that they do not diffuse). The gel is stained for 30 minutes to 1 hour with constant agitation. Save the Coomassie Blue stain to use to stain other gels. Alternatively, the staining may be performed overnight, however, the destaining process will take longer.
30. Destain the gel in two steps: (1) Destain I [50% methanol, 12.5% acetic acid, about 30 minutes]; (2) Destain II [10% methanol, 12.5% acetic acid, for a few hours]. Collect used destain solutions in organic waste containers. When the background staining of the gel is low enough to allow the protein bands to be clearly seen, replace the final destain solution with distilled water. Place the gel with a small amount of liquid into a ziplock plastic bag for storage.

Protocol 3: Purification of Fusion Protein by Affinity Chromatography

The purpose of this protocol is to affinity purify the fusion protein construct with glutathione-agarose.

Materials

- Glutathione agarose, Sigma (1-800-325-3010) catalogue number G4510, 5 ml $16.90.
  In a 50 ml sterile Corning tube, re-suspend the dried Glutathione agarose powder in 50 ml 1X PBS + 0.05% NaN_3. Let the agarose swell overnight at 4°C. Remove the PBS and add 50 ml of fresh PBS. Mix well. Let the Glutathione agarose sit for ~2 hours at 4°C. Repeat this wash and 2 hour storage at 4°C 2 more times. Finally, remove excess PBS to form a 50% slurry (1:1 beads:PBS). Store at 4°C; the hydrated Glutathione agarose can be used for up to 6 months.
  - PBS plus 0.05% sodium azide NaN_3
  - Sonicator

Procedure

Affinity purification of parental GST and ADF1-GST fusion proteins

1. Using the optimum temperature and time course for induction determined in protocol 1, grow cultures to collect the fusion protein. Start with a 2 ml overnight cultures for *E. coli* strains 1 (the gene fusion) and 2 (the pGEX-KG vector). Use these saturated overnight cultures to inoculate 6 ml of LB-AMP medium. It is not necessary to perform an XL1-B (*E. coli* strain 3) control for this experiment.
2. Transfer the cultures to an incubator at the ideal temperature for induction and allow the cells to grow for the appropriate length of time.
3. Transfer the 6 ml cultures into four labeled 1.5 ml microcentrifuge tubes. Pellet the cells by centrifugation at maximum speed in a microcentrifuge for 30 seconds. Remove and discard the supernatant solutions. Resuspend each cell pellet in 200 µl of cold PBS. Pool the replicate samples into a 15 ml Falcon tube.
4. Remove 40 µl of the cells to a labeled tube and make a sample for gel electrophoresis by addition of 40 µl 2X Laemmli sample buffer.
5. Lyse the remaining cells with the sonicator using 3 ten second bursts, interspersed with 10 second rests, to allow the sample to cool. This should be performed on ice to keep the sample from overheating during sonication. Do not allow the sample to froth, as this indicates that protein is being denatured. When the cells are broken, the solution should become translucent. If a sonicator is not available, the cells can be lysed by a series of freeze/thaw cycles.
6. Transfer the bacterial cell lysates to new microfuge tubes. Centrifuge in a microcentrifuge at maximum speed for 15 minutes at 4°C to sediment broken cells and insoluble proteins. Transfer the supernatant solutions to clean microcentrifuge tubes and place the tubes on ice.
7. Mix the glutathione affinity resin thoroughly before withdrawing a sample. Add 80 µl of a 50% slurry to each tube containing supernatant solution.
8. Incubate the resin and samples at room temperature for 30 minutes. Invert the tubes occasional to mix them.
9. Sediment the agarose beads by centrifugation for 30 seconds. Being careful not to disturb the pelleted beads, remove and discard the supernatant solution.

Note: The glutathione agarose forms a very loose, sloppy pellet that is easily disrupted with a micropipetter tip. Use great care so as not to remove beads along with each supernatant solution.

10. Resuspend the beads in 400 µl PBS and vortex briefly. Again pellet the beads by centrifugation. Carefully remove and discard the supernatant solution.
11. Repeat the PBS again.
12. Remove 20 µl of the final pellet of beads and transfer to a fresh tube. Add 20 µl of 2X Laemmli protein sample buffer to this sample, mix by vortexing, and heat the sample to 90-100°C for 5 minutes.

Note: The remaining 60 µl of beads, with bound fusion protein or parental GST, can be stored under a minimal volume of PBS (with sodium azide and protease inhibitors) at 4°C. These beads are used for protease digestion and/or elution with an excess of reduced glutathione. The samples should be used within a few days to a week.

13. Load 10 µl of each sample per lane of a gel. Analyze the proteins by SDS-PAGE followed by Coomassie staining of the gel.

Protocol 4: Elution of fusion protein with glutathione and digestion with thrombin.

Introduction

In this exercise, the intact fusion protein is eluted from the affinity resin (glutathione agarose) by competition with an excess of the substrate, reduced glutathione. Alternatively, the recombinant fusion protein is cleaved into two portions using the protease, thrombin. In this procedure, the GST portion remains attached to the agarose beads and ADF1 plus the polykinker are released into solution. (See Figure 2.)

The pGEX series of plasmid expression vectors are designed with a site-specific protease cleavage site located between the carboxy terminal region of GST and the amino acids encoded by the multiple cloning site (see the GST Gene Fusion Manual). **Thrombin** is a proteolytic enzyme that has a role in blood clotting cascade. It is a serine protease that activates subsequent members of the cascade by cleaving the polypeptide backbone. Thrombin recognizes a specific sequence in the primary structure of polypeptides:

Leu-Val-Pro-Arg-Gly-Ser

and hydrolyzes the peptide bond between adjacent arginine (R) and glycine (G) residues.

In the pGEX-KG vector, a thrombin recognition site (L V P R G S) is located between the carboxy terminus of glutathione S-transferase and the glycine polykinker (see Figure 1). The addition of this flexible, polypeptide linker region facilitates the cleavage of GST moiety from the protein of interest while the fusion is still attached to the affinity matrix (Guan and Dixon, 1991). This allows purification of the ADF1 portion in a single step. The alternative is to elute the intact fusion protein with soluble glutathione, cleave with thrombin, and readsorb the GST portion onto glutathione agarose.

Materials (in addition to items listed in previous protocol).

- Glutathione Elution Buffer: 10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0, freeze in 1 ml aliquots. Sigma (1-800-325-3010) catalogue number G-4251
- Thrombin: Boehringer Mannheim, 1-800-262-4911, catalogue number 620400, dissolved in 100µl PBS.

1 NIH unit=5 cleavage units. A cleavage unit is defined as the amount of enzyme that will completely digest 100 µg of fusion protein in 16 hours at 22°C. Enzyme should be stored in small aliquots (5 µl) at -70°C.

Procedure:

Begin with the isolated GST-ADF1 fusion protein and parental GST protein attached to glutathione agarose obtained from the protocol on affinity purification (Protocol 3). The pellet of agarose beads containing bound fusion protein or parental GST is split into 2 aliquots. One aliquot will be used for thrombin digestion, and the other for elution with glutathione.

A. Thrombin Digestion of Fusion Product Attached to the Glutathione Agarose matrix.

This strategy is generally used when further experiments require the use of a recombinant protein that is not conjugated to the GST polypeptide. For example, if the GST portion would interfere with binding studies, enzymatic activity or antibody production, it is best to remove it by cleaving at the specific protease (thrombin) site located between the GST polypeptide and the peptide from the cloned gene.

NOTE 1: If the amino acid sequence for the protein of interest is known, it is important to examine it for the presence of thrombin sites before proceeding with this method. The presence of additional thrombin recognition sequences will lead to removal of the GST tag but will also result in proteolytic cleavage of the protein.

NOTE 2: If the protein of interest is to be used for subsequent studies where the presence of thrombin might be a detrimental factor, the protease can be removed by adsorption to p-aminobenzamidine Sepharose (Sigma B2768), a protease inhibitor covalently attached to an affinity matrix.

1. The TA/instructor prepares a working dilution of thrombin by diluting the concentrated enzyme 20-fold with cold PBS (i.e. 5 µl of thrombin stock + 95 µl of PBS). Store the thrombin on ice until ready to use.
2. Add 20 µl of diluted thrombin solution to the centrifuge tubes containing 20 µl of PBS-washed beads with attached proteins a) GST-ADF1 fusion and b) GST.
3. Incubate the samples at room temperature for 2 hours.

4. Sediment the beads by centrifugation in a microcentrifuge at maximum speed for 30 seconds. Transfer the supernatant solution to a fresh microcentrifuge tube labeled "thrombin eluate". Add 20 µl of 2X protein sample buffer, vortex and heat the sample at 90-100°C for 5 minutes.
5. Re-suspend the beads in 100 µl of PBS, vortex briefly and sediment the beads using the microcentrifuge.
6. Transfer the supernatant solution to a fresh tube labeled "thrombin wash 1". Add 25 µl of 5X Laemmli protein sample buffer, vortex and heat at 90-100°C for 5 minutes.
7. Repeat this wash two more times, each time with 100 µl of PBS. Label the tubes "thrombin wash 2" and "thrombin wash 3". Prepare the samples for gel electrophoresis as in 7. above.
8. To the agarose beads remaining in each microfuge tube, add 20 µl of 2X Laemmli protein sample buffer, vortex and heat. Mark this tube "post thrombin beads".
9. Load 20 µl of each sample on a 10% SDS-PAGE resolving gel.

   NOTE: A higher percentage gel is required to resolve the small ADF1 polypeptide (16 kDa).
10. Run the gel, Coomassie stain and destain according to the instructions in Protocol 2.

B. Elution of bound ADF1-GST fusion and parental GST from the matrix.

If the presence of a GST moiety attached to the protein of interest is not likely to interfere with functional assays or generation of antibodies, the easiest way to obtain large quantities of purified recombinant protein is by elution with an excess of the substrate, reduced glutathione. In this procedure, the results are analyzed by SDS-PAGE gel electrophoresis. They could also be analyzed by Western immunoblotting. However, if subsequent studies are to be performed with the eluted fusion protein, excess glutathione can be removed by dialysis.

1. To the remaining tube containing 20 µl of affinity matrix with bound ADF1-GST fusion protein or parental GST, add 20 µl of glutathione elution buffer.
2. Incubate the samples at room temperature for 15 minutes with occasional mixing.
3. Sediment the beads by centrifugation in a microcentrifuge at maximum speed for 30 seconds. Transfer the supernatant solution to a fresh microcentrifuge tube labeled "glutathione eluate". Add 20 µl of 2X Laemmli protein sample buffer, vortex the samples and heat at 90-100°C for 5 minutes.
4. Re-suspend the beads in 20 µl of glutathione elution buffer, vortex briefly and sediment the beads using a microcentrifuge.
5. Transfer the supernatant solution to a fresh tube labeled "glutathione eluate 2". Add 20 µl of 2X Laemmli protein sample buffer, vortex and heat at 90-100°C for 5 minutes.
6. Repeat this wash two more times, each time with 100 µl of PBS. Save each of the supernatants in a fresh tube. Label the tubes "glutathione eluate 3 and 4". Prepare the samples for gel electrophoresis as in 3 above.
7. To the agarose beads remaining in the microfuge tube, add 20 µl of 2X Laemmli protein sample buffer, vortex the sample and heat at 90-100°C for 5 minutes. Mark this tube "post-elution beads".

8. Load 20 µl of each sample on a 10% SDS-PAGE gel.
9. Run the gel, Coomassie Blue stain, and destain as before.

Notes to the Instructor

1. Be sure to stress safety precautions to students as they prepare SDS-PAGE gels. Acrylamide is a neurotoxin. It may also be a carcinogen and a mutagen. The greatest hazard from acrylamide occurs when weighing out the powdered acrylamide. Once acrylamide has been polymerized it is far less hazardous. However, a polymerized gel may still contain some unpolymerized acrylamide monomer. Use caution when working with acrylamide. Wear gloves when handling acrylamide. If working with the powder, use a facemask.
2. To minimize the exposure of students to acrylamide:
   a. The instructor may prepare the gels for the students. To store a gel for up to one week, cover with the gel with a paper towel dampened with running buffer, wrap with plastic wrap, and refrigerate. If a resolving and stacking gel that differ in pH are to be used, only the resolving gel should be made in advanced and stored.
   b. Acrylamide can be purchased in liquid solution (as a 30% or 40% solution of acrylamide with bisacrylamide) so one does not have to deal with weighing out the powdered acrylamide.

      30% Acrylamide/Bis Solution, 37.5:1 BioRad (1-800-424-6723) catalog # 161-0158.

      The acrylamide/bis solutions may be stored for 1 year at 4°C. One 500 ml bottle of the 30% acrylamide/bis would be enough to make more than 200-12% mini gels.

   c. Precast gels can be purchased. Precast gels are stored at 4°C and have a 3 month shelf life.

      Ready Gel, 4-15% gradient gel, for the Mini-Protean II gel box BioRad (1-800-424-6723) catalogue number 161-0902.

   d. Agarose has been suggested as an alternative to PAGE gels. Certain agaroses have been used successfully to separate proteins. The use of agarose rather than acrylamide can have certain advantages. First of all, because agarose is non-toxic, it can be used in high school or undergraduate laboratory settings more easily than can acrylamide. Secondly, the removal of an isolated protein from agarose is a simple procedure.

One agarose that has been used successfully to separate proteins is MetaPhor XR agarose from FMC Bioproducts. (191 Thomaston St. Rockland, ME 04841; 800-341-1574, catalog number 50192). FMC reports that a 4% (4 g/100 ml) concentration of MetaPhor XR agarose can be used to separate proteins that range in size from 20 to 200 kDa and a 3% concentration of the agarose can separate proteins that are 100 to 300 kDa. FMC reports using horizontal or vertical gels for the protein separations.

3. Troubleshooting: If students are casting their own PAGE gels and find that their gels are not polymerizing in a reasonable period of time, there may be several sources of problems.
   a. The presence of oxygen can inhibit the polymerization reaction. Students may mix the components too vigorously and introduce excess oxygen into the reaction.

b. The solutions of TEMED or AMPS may go bad with time. Increase the amount of these used or replace them with fresh solutions.

4. Destaining: To speed the rate of destaining and decrease the changes of destain solution required, place a tissue, such as a Kimwipe, or a piece of Styrofoam, in a corner of the staining tray. Replace the tissues or Styrofoam when they are saturated with Coomassie Blue.
5. Figure 3 shows an idealized sketch of typical student results of an SDS-PAGE gel of samples collected in Protocol 1. Note that these samples contain total *E. coli* proteins. There are numerous proteins observed. There is an endogenous protein that is approximately 45 kilodaltons. There are 45 kilodalton bands present in all lanes, including the lanes from samples of bacteria without plasmid. The induction of the fusion protein or the GST protein can be observed above these background bands. The fusion protein is 45 kilodaltons and the GST protein from the pGEX-KG vector is 26 kilodaltons. These results indicate that the optimum temperature for the induction of fusion protein is 30°C and that more fusion protein is observed at 3 hours after induction than at earlier times.
6. As an alternative to using the ADF fusion protein, a gene of interest can be cloned into the pGEX-KG expression vector to obtain a different fusion protein to study. Alternatively, students can study the induction of the GST tag from the pGEX-KG vector.
7. The equipment needed for this lab module includes (per group of 2 or 3 students): a set of micropipettors (i.e. Rainin P20, P200, and P1000 pipettors); vertical gel apparatus (i.e. BioRad Mini-Protean II gel box); electroblotting apparatus; and a microcentrifuge.

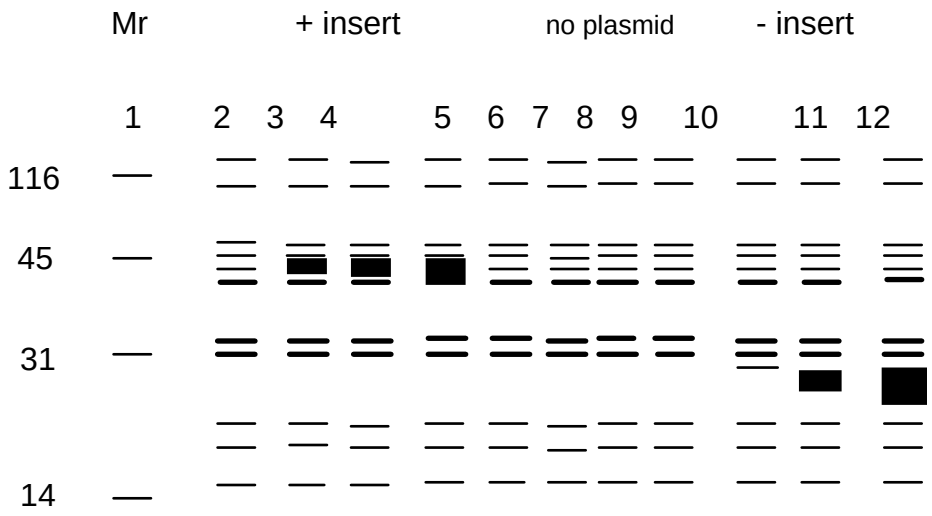

Figure 3. An idealized sketch of results of an SDS-PAGE gel of samples collected in Protocol 1--Determination of optimal time and temperature conditions for overexpression of the fusion protein.

Lane 1: Molecular weight standards. Proteins of known molecular weight are run as size standards. The sizes indicated are in kilodaltons. Lanes 2-6: +insert. Total proteins isolated from *E. coli* containing the gene of interest (ADF1) cloned into the pGEX-KG vector. Note the presence of a 42 kilodalton band which is more intense in some lanes. This is the fusion protein (16 kilodalton ADF1 plus 26 kilodalton GST.) There is an endogenous *E. coli* protein of this size, but in addition, the induction of the fusion protein is observed. Lane 2: 0 hours after addition of the inducing agent, IPTG; growth at 30°C. Lane 3: 1 hour after addition of IPTG, growth at 30°C. Lane 4: 2 hours after addition of IPTG, growth at 30°C. Lane 5: 3 hours after addition of IPTG, growth at 30°C. Lane 6: 3 hours after addition of IPTG, growth at 25°C. Note there is only a faint band at 42 kilodaltons, indicating these conditions do not induce expression of the fusion protein. Lanes 7-9: No plasmid. Total proteins isolated from *E. coli* without a plasmid. Lane 7: 0 hour after addition of IPTG, growth at 30°C. Lane 8: 2 hours after addition of IPTG, growth at 30°C. Lane 9: 3 hours after addition of IPTG, growth at 30°C. Lanes 10-12: -insert. Total proteins isolated from *E. coli* containing the pGEX-KG vector. Note the presence of the 26 kilodalton GST protein. Lane 10: 0 hour after addition of IPTG, growth at 30°C. Lane 11: 2 hours after addition of IPTG, growth at 30°C. Lane 12: 3 hours after addition of IPTG, growth at 30°C.

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